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13. ABSTRACT ***(Maximum 200 Words)***

In this report we present data in support of **Aim 1** of our project. Utilizing human breast carcinoma cell lines as an in vitro model for naturally occurring malignancy, we demonstrated that both the apoptotic and translational machinery are activated in five breast carcinoma cell lines. Suppression of cap-dependent translation by pharmacological inhibitors of 4E-BP1 phosphorylation or by ectopic expression of 4E-BP1 stimulated apoptosis and abrogated chemoresistance in fibroblasts and breast carcinoma cells expressing oncogenic Ras. Activation of apoptosis by translational inhibitors paralleled their ability to repress the cap-dependent translation apparatus. These results show that the integrity of the cap-dependent translational apparatus is critically important for breast cancer cell chemoresistance. They also suggest that targeted disruption of the cap-binding complex can be used as a novel approach for blocking malignant progression in breast carcinoma and other tumors whose growth depends on activation of cap-dependent translation.

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Introduction

Eukaryotic translation initiation factor 4E (eIF4E) is the mRNA cap binding protein which functions during translation of cellular mRNAs possessing the 5′ cap structure. Overexpressed eIF4E suppresses oncogene-dependent apoptosis, causes malignant transformation and leads to multi-drug resistance. The function of eIF4E is negatively regulated by members of the family of translational repressors, the eIF4E-binding proteins 4E-BPs. When hypophosphorylated, 4E-BPs block cap-dependent translation by sequestering eIF4E in a translationally inactive complex. Upon hyperphosphorylation in response to hormones or growth factors, the 4E-BPs and eIF4E dissociate allowing eIF4E to form an active translation initiation complex. Previously, we demonstrated that 4E-BP1 sensitizes fibroblasts to apoptosis and suppresses Ras-dependent tumorigenicity in a manner strictly dependent on its ability to sequester eIF4E from a translationally active complex with eIF4G. The objective of this awarded project is to experimentally test the idea that targeted disruption of the anti-apoptotic function of eIF4E can sensitize breast carcinoma cells to therapeutic doses of a non-genotoxic cytostatic agent such as lovastatin and/or to low concentrations of genotoxic agents. We also propose to develop treatment strategies which will include disruption of the anti-apoptotic function of eIF4E in a combination with treatments with non-toxic doses of lovastatin and conventional anti-neoplastic agents. Here we present data in support of Aim 1 (Task 1) of our project.

Aim I: *In vitro*, examine whether levels of eIF4E expression determine chemoresistance in human breast cancer cell lines.

A. Determine whether susceptibility to drug-induced apoptosis in different human breast cancer cell lines correlates with cellular levels of eIF4E protein.

B. Develop breast cancer cell lines with increased or decreased function of eIF4E and determine:

1. Whether constitutively or inducibly overexpressed eIF4E will suppress apoptosis in drug-susceptible breast carcinoma cell lines. 2. Whether breast cancer cell chemoresistance can be abrogated by a reduction of eIF4E function following ectopic expression of: (a) eIF4E antisense RNA; (b) eIF4E repressor protein 4E-BP1.

Experimental Data in Support of Aim 1

Aim 1A. Determine whether susceptibility to drug-induced apoptosis in different human breast cancer cell lines correlates with cellular levels of eIF4E protein.

Although gain and loss of translation initiation activity potently modulates viability in Myc- and Ras-transformed rodent fibroblasts in our published studies, it remains an open question whether translational control is relevant to regulation of apoptosis in naturally occurring breast cancer cells which are of epithelial origin and posses diverse tumor-related gene alterations. To detect whether susceptibility to spontaneous and drug-induced apoptosis in different human breast cancer cell lines correlates with activity of the cap-dependent translational machinery, protein drug-induced apoptosis assays have been performed in a set of human breast carcinoma cell lines (Table 1), the genetic profile of which have been documented previously (Sepp-Lorensino et al., 1995).

Table 1. Cell lines in use in our studies

Cell line	Ras status	Other characteristics	Cell line source
HMEC 184 A1	wt	Immortalized breast epithelial cells	Berkeley National Laboratory
MDA-MB-231	Ki-V12	ER-	ATCC
MDA-MB-453	wt	ER-/erbB2+++/MAPK+++	ATCC
MCF-7	wt	ER+/IGF-IR +++	Dr. Yee, UM Cancer Center
MDA-MB-468	wt	ER-/EGFR+++	Dr. Yee, UM Cancer Center
SkBr-3	wt	ER-/HER2+++	Dr. Yee, UM Cancer Center

The Ras status, estrogen (ER) dependence, and other characteristics of non-transformed breast epithelial cells and breast carcinoma lines are shown. One cancer cell line (MDA-MD-231) harbors mutated Ki-Ras, while others express activated upstream effectors of eIF4E signaling pathways.

Extracts from non-transformed breast epithelial cells and breast cancer cells lines were tested to detect cellular levels of eIF4E and 4E-BP1, and to evaluate the association of eIF4GI with eIF4E to form an intact translation initiation complex (Figure 1). Steady state levels of eIF4E were similar among the breast cancer cell lines and only modestly increased compared to the non-transformed 184 A1 breast epithelial cells (Figure 1a). In contrast, steady state levels of eIF4G1 were significantly increased in all breast cancer cell lines tested. While 4E-BP1 is predominantly represented in non-transformed cells by hypophosphorylated isoform α which actively represses translation, breast cancer cell extracts are enriched with slow migrating hyperphosphorylated 4E-BP1 (isoforms β and γ) which is much less active in repressing assembly of eIF4F. Consistent with this, breast cancer cell extracts manifested increased amounts of eIF4GI associated with eIF4E in the cap bound fraction (Figure 1b). This indicates increased amounts of intact eIF4F complex in all breast cancer cell lines tested, suggesting these cells function in a translationally activated state.

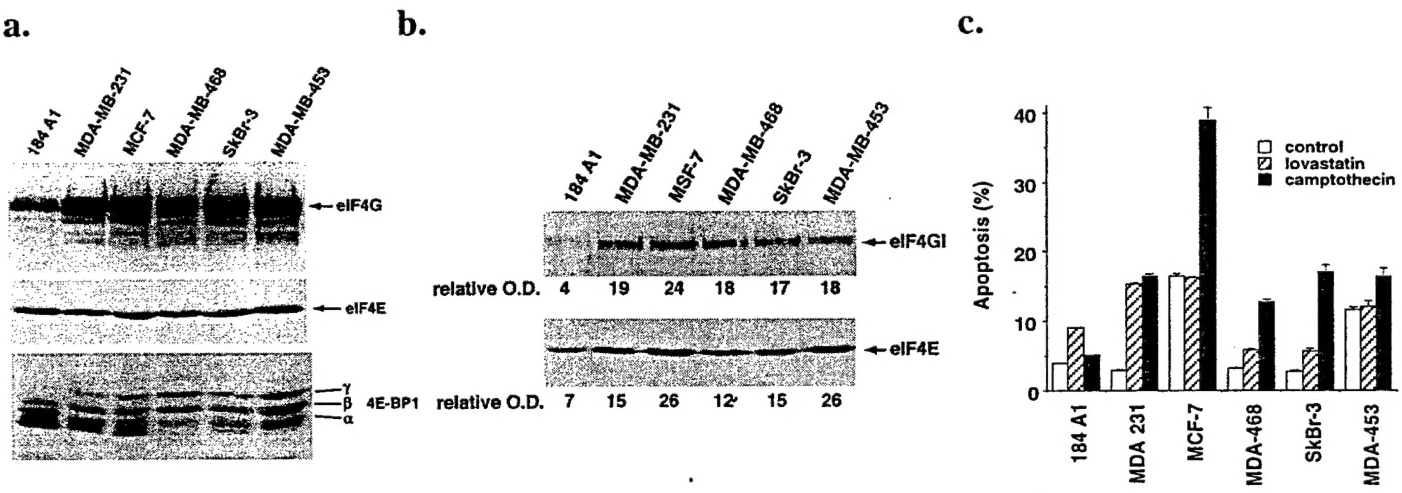

Figure 1. Selective activation of the eIF4F translation complex in breast cancer cells. **(a)** Western blot of cellular eIF4E, eIF4G1, and 4E-BP1. **(b)** Immunoblot analysis of eIF4GI associated with cap-bound eIF4E. For the cap-affinity assay, cell lysates (250 ug) were incubated with 7-methyl-GTP Sepharose resin (Amersham Pharmacia Biotech) to capture eIF4E and its binding partners. Samples were eluted with buffer containing 70 μM 7-methyl-GTP. Cap bound material was subjected to SDS PAGE and transferred to nitrocellulose. Blots were probed for eIF4E (mouse monoclonal antibody, 1:500, Transduction Laboratories), and for eIF4GI (rabbit polyclonal antibody, 1:4000). **(c)** Cells were incubated in the presence of 7.5 μM lovastatin or 500 nM camptothecin for 24 h, and percentages of cell with hypodiploid DNA contents (% of apoptosis) were quantified by flow cytometry Each bar represents the mean ± SD (4 independent replications)..

Apoptosis assays revealed elevated spontaneous apoptosis in MCF-7 and MDA-MB-453 cells as well as increased susceptibility to drug-induced apoptosis in all tested breast carcinomas (Figure 1c). Since upregulated cap-dependent translation antagonizes apoptotic death (Polunovsky et al, 1996; Tan et al., 2000), we hypothesized that breast cancer cells require a high level of cap-dependent translation to suppress the apoptotic apparatus that is activated in the course of cell malignant transformation.

Aim 1B. Develop breast cancer cell lines with increased or decreased function of eIF4E …

Enforced expression of 4E-BP1 stimulates spontaneous and drug-induced apoptosis in MDA-MB-231 cells. To test this hypothesis, we examined the effects of inhibitors of cap-dependent translation on spontaneous and drug-induced apoptosis in normal and malignant cells. Since breast cancer cells significantly differ from normal epithelial cells in expression of 4E-BP1 (Figure 1A), we focused first on developing cell lines in which cap-dependent translation is inhibited by enforced overexpression of 4E-BP1. Our published data (Polunovsky et al., 2000) show that translational repressor 4E-BP1 and 4E-BP1 phosphorylation inhibitor rapamycin sensitized transformed fibroblasts to apoptosis and suppresses Ras-dependent tumorigenicity in a manner strictly dependent on their ability to sequester eIF4E from a translationally active complex with eIF4G. To determine whether the anti-apoptotic effect of 4E-BP1 observed in Ras-transformed fibroblasts is also seen in breast cancer cells, MDA-MB-231 cells expressing oncogenic Ras were stably transfected with a *neo* selectable vector pACTAG-2 engineered to encode haemagglutinin (HA) tagged human wt 4E-BP1(Gingras et al., 1999).. Twelve neomycin-resistant clones were isolated and assayed for steady state expression of HA; subjected to cap-affinity chromatography to quantify the proportion of eIF4E complexed with eIF4GI and examined for chemosensitivity to lovastatin and camptothecin (Figure 2). In the three clones shown (Figure 2a), HA expression (as a surrogate for ectopic 4E-BP1), eIF4E captured by cap-analog, and eIF4GI associated with cap-bound eIF4E can be

compared to these parameters in non-transfected MDA-MB-231 cells. In accord with expectation, ectopic 4E-BP1 displaced eIF4GI from eIF4E (Figure 2b). This was accompanied by an increase in intrinsic apoptosis and substantially augmented apoptotic death in response to the lovastatin or camptothecin (Figure 2c).

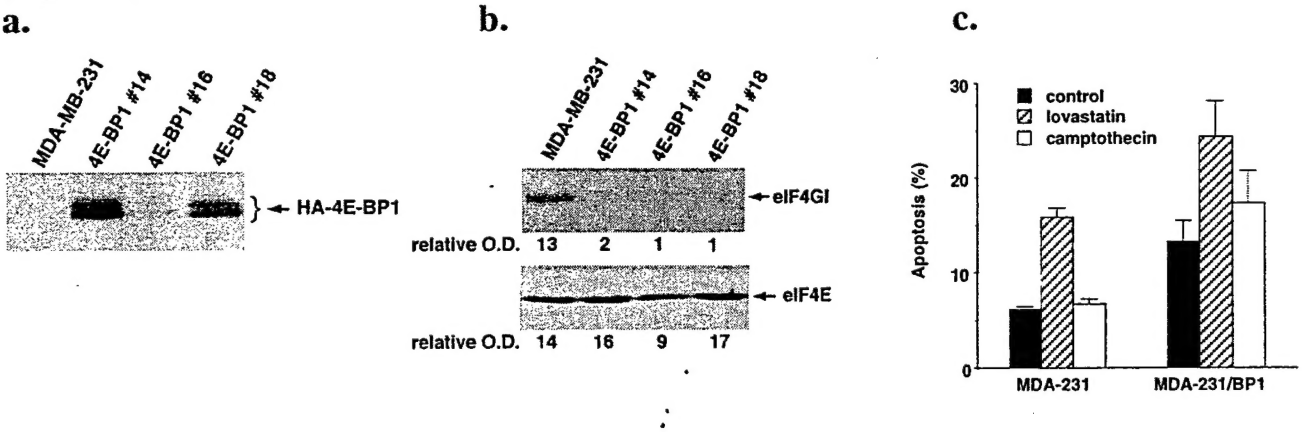

Figure 2. 4E-BP1 displaces eIF4G1 from eIF4E and sensitizes MDA-MB-231 cells to apoptosis. (**a,b**)Immunoblot analysis of HA-4E-BP1 expression (**a**) and binding of eIF4G1 to the cap-captured eIF4E (**b**), in three clonal cell lines of MDA-MB-231 transfected with a construct encoding HA-4E-BP1. (**c**) Summative analysis of apoptotic frequencies in twelve MDA-MB-231 clonal cell lines ectopically expressing 4E-BP1. Cells were cultured for 24 h +/- 7.5 μM lovastatin or 200 nM camptothecin. Apoptosis was quantified by flow cytometry. Each bar represents the mean ± SD (3 independent replications).

To confirm activation of apoptosis-related biochemical events in 4E-BP1 expressing cells and independently verify the results of flow cytometry, we used immunomorphological techniques in which we identify nuclear apoptotic rearrangements along with expression of active capspase-3 (Figure 3).

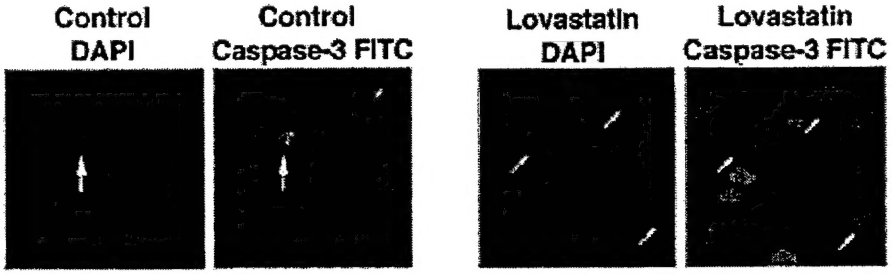

Figure 3 Morphological hallmarks of apoptosis and caspase-3 activity in MDA-MB-231 breast cancer cells ectopically expressing wild type 4E-BP1 (BP1-wt). Cells were incubated in the presence or absence of 7.5 uM lovastatin for 24h and immunostaining for active caspase-3 and staining of nuclei with DAPI was carried out. Arrows highlight apoptotic cells and demonstrate that cells with apoptotic nuclei are caspase-3 positive, consistent with the order of events during apoptosis.

Discussion

Here we present data in support of **Aim 1.**

1. We found that both the apoptotic and translational machinery are activated in all tested breast carcinomas when compared to non-transformed breast epithelial cells. Together with our observations that activated cap-dependent translation can rescue cells from apoptotic death

(Polunovsky et al., 1996, Tan et al., 2000), these findings demonstrate proof of principle: breast cancer cells acquire metabolic alterations leading to increased cap-dependent translation to oppose transformation-related activation of their intrinsic apoptotic program.

2. Contrary to our expectations (see Aim 1A) and published data (reviewed by Zimmer et al., 2000), transformation-related increase in cap-dependent translation in breast epithelial cells was not associated with elevated expression of translational factor eIF4E. Instead, breast carcinomas revealed increased phosphorylation of the translational repressor 4E-BP1 and enhanced production of translation factor eIF4G1. These results suggest that alterations in steady state levels of eIF4E are not main determinants of activating cap-dependent translation and chemoresistance in breast cancer cells as previously believed. They also indicate that at least in breast carcinomas, normalization of the upregulated apoptotic machinery can be achieved more effectively by disrupting association of eIF4E with eIF4G than by blocking overproduction of eIF4E.

3. In accord with our expectations, inhibition of 4E-BP1 phosphorylation by rapamycin or ectopic expression of wild type 4E-BP1 stimulates apoptosis and abrogates chemoresistance in fibroblasts and breast carcinoma cells expressing oncogenic Ras. Activation of apoptosis by translational inhibitors parallels their ability to disrupt eIF4E/eIF4G assembly and repress function of the cap-dependent translation apparatus. These results demonstrate that targeted disruption of the cap-binding complex can be used as a novel approach to blocking malignant progression in breast carcinoma and other tumors whose growth depends on activation of cap-dependent translation.

Key Research Accomplishments

• Based on our findings, we argue that disruption of signaling pathways that activate eIF4E is a more effective tool for inhibition of cap-dependent translation in breast carcinomas than decreasing cellular eIF4E production.

• Our results suggest that transfer of genes encoding translational repressors that operate at the apical step of cap-dependent translation initiation will selectively chemosensitize breast cancer cells to safe doses of HMG-CoA reductase inhibitors and conventional chemotherapy without harming desirable non-transformed bystanders.

• Our data open a novel approach to activate apoptosis in malignant cells by disruption of 4E-BP1 phosphorylation. It might be achieved by expressing hypophosphorylated mutants of 4E-BP1 and/or by pharmacological inhibition of the upstream signaling that leads to 4E-BP1 phosphorylation.

• Our results demonstrate the feasibility of synthesizing and screening novel anti-cancer therapeutics that are focused on inhibition of cap-dependent translation initiation - a novel target regulating an apoptotic pathway that we have discovered. Based on these results, we submitted to NIH our new research project "Translational apparatus as a target for cancer drug discovery" (RFA: CA-00-002) in which we propose to experimentally test the hypothesis that phosphoramidated cap derivatives will activate apoptosis in a wide spectrum of cancer cells with an upregulated translational apparatus.

Reportable Outcomes

• Manuscripts, Abstracts, Presentations

Manuscript:

1. Avdulov S, **Shunan Li**, Peterson M, Gingras A-C, Sonenberg N, Bitterman P, and **Polunovsky VA,. Translation repressor 4E-BP1 activates apoptosis in breast cancer cells in a manner dependent on its phosphorylation status. Cancer Res., (In preparation).**

Abstracts:

1. Shunan Li, Peterson M, Murray J, Schwarze J, Bitterman P and **Polunovsky VA** Activation of apoptosis by translational repressor 4E-BP1 depends on its phosphorylation status. ASCB Annual Meeting, December 9-13, San Francisco, 2000.
3. **Polunovsky VA,.** Avdulov S, Shunan Li, Peterson M, Gingras A-C, Sonenberg N, and Bitterman P. Translation control of malignancy: Translation repressor 4E-BP1 activates apoptosis in breast cancer cells in a manner dependent on its phosphorylation status. AACR Annual Meeting,, Mach 24-28, New Orleans, LA , 2000 (Submitted)

Presentations:

1. Takasu T, Bitterman PB, **Polunovsky VA.** Translation factor eIF4E rescues Myc overexpressing cells from drug-induced apoptosis through a Bcl-XL-mediated blockage of caspase-3 activation. Abstract was selected for oral presentation at Cold Spring Harbor Laboratory Meeting"Programmed Cell Death", September-October 1999, Cold Spring Harbor, NY, 1999.
2. **Polunovsky VA,** Bitterman P, Takasu T. Translation factor eIF4E operates through Bcl-X_L to suppress Myc-dependent apoptosis. Abstract was selected for oral presentation at ASCB Annual Meeting, December 11-15, Washington DC,1999

• Patents

Bitterman PB, **Polunovsky VA**, Sonenberg N, Gingras A-C. Methods of Modulating Pro-Apoptotic and Anti-Apoptotic Pathways in Ras-transformed Cells.
NIH Invention Disclosure Number 1450401-98-0033

• Degrees obtained that are supported by this research

Not available

• Informatics such as databases and animal models, etc

Not available

• Funding applied for based on work supported by this award

Active:

The Twin Cities Affiliate of Susan G. Komen Breast Cancer Foundation (**V. Polunovsky PI**)

The Pro-Apoptotic Function of Translational Repressor 4E-BP1 in Breast Carcinomas.
03/15/2000-03/14/2001 $22,500, 0% effort
Pilot study of in vitro

Pending:

National Institutes of Health

Translational Apparatus as a Target for Cancer Drug Discovery (**V. Polunovsky** PI)
RFA CA-00-002
04/01/2001-03/31/2005 350,000 Direct cost/year, 40% effort

• **Employment or research opportunities applied for/or received....**
Not available

Conclusions

In this report we present experimental data in support of our hypothesis that levels of expression of the translational factor eIF4E determine chemoresistance in human breast cancer cell lines (Aim 1). Our results clearly show that both the apoptotic and translational machinery are activated in human breast carcinomas. Surprisingly, we found no significant changes in expression levels of eIF4E compared to normal breast epithelial cells. Instead, breast carcinomas revealed enhanced production of translation factor eIF4G1 and increased phosphorylation of the translational repressor 4E-BP1 which blocks association eIF4E with eIF4G. In line with these observations, ectopically expressed 4E-BP1 stimulates spontaneous apoptosis and abrogates chemoresistance in the breast carcinoma cell line which harbored oncogenic Ras. Thus, we concluded that alterations in steady state levels of eIF4E are not main determinants in activating cap-dependent translation in breast carcinoma. More likely is that breast cancer cell chemoresistance is determined by alterations in expression levels of eIF4G and by activity of upstream regulators implicated in control of association between eIF4E and eIF4G. Most importantly from a therapeutic point of view, we found that disruption of the translational complex assembly specifically activates apoptosis in cancer cells, but not in non-transformed cells (Polunovsky et al., 2000), identifying the translational complex as a potential novel molecular target for anticancer drug discovery. Based on these results, we plan to focus our future studies on genetic and pharmacological interventions leading to normalization of upregulated apoptotic machinery in malignant cells by disrupting the cap-dependent translational complex.

The observations obtained based on this award prompted us to submitted a new NIH grant proposal "Translational apparatus as a target for cancer drug discovery" (RFA: CA-00-002, V. Polunovsky PI). In this project, we propose to synthesize and test in vitro phosphoramidate derivatives of 7'methyl guanosine nucleotide analogues with favorable drug-like properties, designed to repress cap-dependent translation initiation. We also plan to utilize preclinical models of breast and lung cancer to test the most promising compound for its ability to collaborate with well-tolerated doses of available cancer therapeutics to inhibit xenograft growth in athymic mice.

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Tan A, Bitterman P, Sonenberg N, Peterson M, and Polunovsky V. Inhibition of Myc-dependent apoptosis by eukaryotic translation initiation factor 4E requires cyclin D1. *Oncogene* 2000, *19*: 1437-1447.

Zimmer SG, De Benedetti A, and Graff J. Translation control of malignancy: the mRNA cap-binding protein, eIF-4E, as a central regulator of tumor formation, growth, invasion, and metastasis. *Anticancer Res* 2000, 20: 1343-1352

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License to the United States Government

Invention Title: Method of Modulating Proapoptotic and Antiapoptotic Pathways in Ras-transformed Cells

Inventor(s): Nahum Sonenberg, Anne-Claude Gingras, Vitaly A. Polunovsky, Peter B. Bitterman

Patent or Application Serial No.: 60/168,398

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Foreign Applications filed/intended in (countries): Undecided

The invention identified above is a Subject Invention under **35 U.S.C. 200, et seq.**, and the Standard Patent Rights clause at **37 CFR 401.14 or FAR 52.227-11**, which are included among the terms of the above-identified grant/contract award from the Public Health Service/National Institutes of Health. This document is confirmatory of:

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(Grantee/Contractor Official and Title)
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For Regents of the University of Minnesota
(Organization)

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Verify Update Invention Disclosure Screen

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- translation

Inventors' Names:
- Bitterman, Peter B
- Polunovsky, Vitaly A
- Sonenberg, Nahum
- Gingras, Anne-Claude

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